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Simple and rapid HPLC method for simultaneous determination of atenolol and chlorthalidone in spiked human plasma

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Abstract A simple, sensitive and rapid chromatographic method was developed and validated for the simultaneous quantification of atenolol and chlorthalidone in human plasma using hydrochlorothiazide as internal standard (IS). The method utilized proteins precipitation with acetonitril as the only sample preparation involved prior to reverse phase-HPLC. The analytes were chromatographed on Shim-pack cyanopropyl column with isocratic elution with 10 mM KH₂PO₄ (pH 6.0) – methanol (70:30, v/v) at ambient temperature with flow rate of 1 mL min⁻¹ and UV detection at 225 nm. The chromatographic run time was less than 10 min for the mixture. The calibration curves were linear over the range of 0.1–10 µg mL⁻¹. The method was validated in terms of accuracy, precision, absolute recovery, freeze–thaw stability, bench-top stability and re-injection reproducibility. The within- and between-day accuracy and precision were found to be within acceptable limits < 15%. The analytes were stable after three freeze–thaw cycles (deviation < 15%). The proposed method was specific for the simultaneous determination of atenolol and chlorthalidone in human plasma where there was no interference from endogenous biological substances.

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1. Introduction

Atenolol is a β_1 -selective β -adrenergic receptor-blocking agent and clinically used for the treatment of hypertension. Like other antihypertensive drugs, atenolol lowers the systolic and diastolic blood pressure by 15–20% in a single drug treatment and reduces cardiovascular mortality. It is also used alone or in combination with other antihypertensive agents for the treatment of myocardial infarction, arrhythmias, angina and disorders arising from decreased circulation and vascular constriction, including migraine (Prichard et al., 2001; Wadworth et al., 1991). Atenolol is rapidly but incompletely absorbed

after oral administration. It is excreted almost entirely as unchanged drug, 35–50% of an oral dose being excreted in the urine and 30–50% in the faeces in 24 h. After a single oral dose of 100 mg given to 12 subjects, peak plasma concentrations of $0.41\text{--}0.87\text{ mg}^{-1}\text{ L}$ (mean 0.6) were attained in about 3 h (Moffat et al., 2004).

Chlorthalidone is a diuretic drug widely used in hypertension therapy. This sulphonamide-like diuretic differs chemically from the thiazides by the nature of the heterocyclic ring, although its pharmacological action is indistinguishable from that of the thiazides (Thomson, 1970). Chlorthalidone is readily but incompletely absorbed after oral administration. It does not appear to be significantly metabolized. After a single dose, about 25–40% is excreted in the urine as unchanged drug and about 1% is eliminated in the bile; the quantity excreted in the urine appears to be dose-dependent. During daily therapy, about 50% of the daily dose is excreted unchanged in the urine in 24 h and about 25% is eliminated in the faeces. After single oral doses of 50–75 mg given to 7 subjects, peak plasma concentrations of $0.14\text{--}0.26\text{ mg/L}$ were attained in 1–3 h (Moffat et al., 2004).

In recent years, pharmaceutical preparations containing both drugs have also been marketed, their combination having the advantage of providing greater therapeutic effects than with either drug alone or permitting once a day administration only, the two drugs not interacting pharmacokinetically with each other or presenting synergic toxic effects. The two drugs are of limited metabolism and eliminated unchanged from human body (Sweetman, 2006).

In open literature, several methods have been reported for the determination of atenolol in biological fluids that relied on HPLC with UV or fluorimetric detection (Guadrado et al., 2002; Martins et al., 1997; Niopas et al., 2000; Ranta et al., 2002; Wu et al., 2003), HPLC with MS detection (Dong and Huang, 2006; Johnson and Lewis, 2006; Dupuis et al., 2004), and adopting gas chromatographic techniques with an electron capture detector (Malluca and Monson, 1975; Wan et al., 1978).

On the other hand, chlorthalidone has been measured in biological fluid both by GC and nitrogen detection (Degen and Schweizer, 1977; Ervik and Gustavii, 1974), HPLC using analogous columns and UV detection (Dadgar and Kelly, 1988; Muirhead and Christie, 1987), or HPLC coupled with MS detector (Yu et al., 2005; Goebel et al., 2004). The previously reported methods applied liquid-liquid extraction or solid phase extraction as the main technique and there is no method applied for protein precipitation technique for their determination in biological fluids.

Atenolol and chlorthalidone mixture is officially listed in USP. To our knowledge, there are many methods that have been published for simultaneous determination of both drugs in pharmaceutical formulations like chemometric-assisted spectrophotometric (Ferraro et al., 2003; Mohamed and Salem, 2005; El-Gindy et al., 2005) and HPLC with UV detection (El-Gindy et al., 2005). These methods were not suitable for simultaneous determination of the proposed mixtures in biological fluids due to matrix interference. Recently our laboratory applied chromatographic technique for simultaneous determination of ternary mixtures for hypertension treatment containing the two studied drugs (Elshanawane et al., 2009). This method failed to quantitate the studied mixture in plasma because of the short retention time of atenolol that interfered with plasma

matrix (Elshanawane et al., 2009). Additionally there are two methods that have been described for simultaneous determination of both drugs in human milk (El-Gindy et al., 2008) and plasma (Giachetti et al., 1997). These methods required long time for preparation of samples or relied on tedious liquid-liquid extraction procedure. Therefore, it was desirable to develop simple, accurate, and fast procedure that could be applied for the simultaneous determination of atenolol and chlorthalidone in plasma after protein precipitation as the only sample preparation step involved prior to reversed phase-HPLC.

2. Experimental

2.1. Chemicals and reagents

Atenolol (Almirall prodes farma, Spain, 99.8%), Chlorthalidone (Changzhou Zinhua Chemical product Co. Limited, China, 99.85), and hydrochlorothiazide (IS) (Aurbindo Ltd., Ireland, 99.9%) were used. Methanol and acetonitril (HPLC grade) were purchased from (Riedel-de Haen laboratory chemicals, Germany). Potassium dihydrogen phosphate, phosphoric acid, and NaOH (Sigma-Aldrich, Inc., St. Louis, USA) used were of analytical grade. Double distilled water was used throughout the study.

2.2. Equipment

The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with a model series LC-10 ADVP pump, SCL-10 AVP system controller, DGU-12 A Degasser, Rheodyne 7725i injector with a 20 μL loop and a SPD-10AVP UV-VIS detector. The sample was injected with 25 μL Hamilton analytical syringe. Data acquisition was performed on Class-VP software (version 6).

2.3. Chromatographic condition

The HPLC separation and quantitation were achieved on a $250 \times 4.6\text{ mm}$ (I.d) Shim-pack® (5 μm particle size) cyanopropyl column (Shimadzu, Kyoto, Japan). The mobile phase was prepared by mixing 10 mM KH_2PO_4 (pH 6.0) – methanol (70:30, v/v) that run isocratically at flow rate 1 mL min^{-1} . All determinations were performed at ambient temperature. The injected volume was 20 μL . The detector was set at λ 225 nm.

2.4. Preparation of standard and quality control samples

Stock standard solutions of the studied drugs in the two mixtures (1 mg mL^{-1}) were separately prepared in methanol. Working solutions were prepared by appropriate dilution in methanol just before use. All solutions were stored in darkness at 4°C . Volumes of 20 μL of the preparing working solutions and 20 μL of IS solution ($150\text{ }\mu\text{g mL}^{-1}$) were added to 960 μL of drug-free human plasma to obtain drug concentration levels of 0.1, 0.2, 0.6, 1.0, 2.0, 5.0, 7.0, and $10\text{ }\mu\text{g mL}^{-1}$. Quality control (QC) samples were prepared separately and pooled at three different concentration levels (0.1, 1, $10.0\text{ }\mu\text{g mL}^{-1}$) as low, medium and high, respectively. The samples were stored in a freezer at -20°C until analysis. A calibration curve was constructed from a blank sample (a plasma sample processed

without the IS), a zero sample (a plasma processed with the IS) and eight non-zero samples covering the total range of 0.1–10.0 $\mu\text{g mL}^{-1}$, including the LOQ. Calibration curves were generated using the analytes to IS peak area ratios by least-squares linear regression.

2.5. Sample preparation

The stored plasma samples were thawed at room temperature before processing. The plasma samples were centrifuged at 4000 rpm for 10 min. An aliquot (1.0 mL) was pipetted into a 10 mL polypropylene tube and acetonitril (2.0 mL) was added. The mixture was vortex mixed briefly, and after standing for 5 min at room temperature, the mixture was centrifuged at 4000 rpm for 20 min and the supernatant (20 μL) was injected into HPLC system.

2.6. Validation of the bioanalytical method

The method was validated by the determination of the following parameters: specificity, linearity, range, recovery, accuracy, precision, lower limit of quantitation (LLOQ), and stability studies according to the currently accepted US food drug administration (FDA) bioanalytical method validation guidance (US Department of Health and Human Services, 2001).

2.7. Specificity

Randomly selected six blank human plasma samples, which were collected under controlled conditions, were carried through the protein precipitation procedure and chromatographed individually to determine the extent to which endogenous plasma components could contribute to interference with the analyte or the internal standard.

2.8. Calibration curve

The calibration curves were constructed from a blank sample (a plasma sample processed without IS), a zero sample (a plasma processed with IS), and eight concentrations of the studied drugs including the LLOQ, ranging from 0.1 to 10 $\mu\text{g mL}^{-1}$. The peak area ratio of the drug to the IS against the respective standard concentrations was used for plotting the graph and the linearity evaluated by a least squares regression analysis.

2.9. Recovery

The analytical recovery was calculated by comparing chromatographic peak areas from standard samples prepared post-extracted blank plasma and from standard spiked samples at three different concentrations (0.1, 1, 10.0 $\mu\text{g mL}^{-1}$) for the studied drugs.

2.10. Accuracy and precision

To evaluate the inter-day precision and accuracy, the quality control samples were for 3 consecutive days, while intra-day precision and accuracy were evaluated through analysis of validation control samples at three different concentrations in six replicates in the same day. Inter- and intra-day precision was expressed as relative standard deviation (RSD). The accuracy was expressed as the relative error (RE) for the determination

of the studied drugs in each human plasma sample. The evaluation of precision was based on the criteria (US Department of Health and Human Services, 2001) that the deviation of each concentration level should be within $\pm 15\%$, except for the LLOQ, for which it should be within $\pm 20\%$. Similarly for accuracy, the mean value should not deviate by $\pm 15\%$ of the nominal concentration, except the LLOQ, where it should not deviate by $\pm 20\%$ of the nominal concentration.

2.11. Lower limit of quantification (LLOQ) and limit of detection (LOD)

Lowest standard concentration on the calibration curve should be accepted as the limit of quantification if the following conditions are met: the analyte response at the LLOQ should be at least five times the response compared to blank response and analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80–120%. The limit of detection (LOD) was defined by the concentration with a signal-to-noise ratio of 3.

2.12. Stability

The concentration of the studied drugs after each storage period was related to the initial concentration as zero cycle (samples that were freshly prepared and processed immediately). The samples were considered stable if the standard deviation (expressed as percentage bias) from the zero cycle was within $\pm 15\%$.

2.12.1. Freeze–thaw stability

The freeze–thaw stability of the studied drugs was determined at low, medium, and high QC samples ($n = 3$), over three freeze–thaw cycles within 3 days. In each cycle, the frozen plasma samples were thawed at room temperature for 2 h and refrozen for 24 h. After completion of each cycle the samples were analyzed and the results were compared with that of zero cycle.

2.12.2. Short-term stability (bench-top stability)

Three aliquots each of the low, medium, and high unprocessed QC samples were kept at room temperature for 24 h. After 24 h the samples were analyzed and the results were compared with that of zero cycle.

2.12.3. Long-term stability

Three aliquots each of the low, medium, and high QC samples were frozen at $-20\text{ }^{\circ}\text{C}$ for 30 days. The samples were analyzed and the results were compared with that of zero cycle.

3. Results and discussion

To obtain the best chromatographic condition, different columns like C18, C8, and CN-propyl and mobile phases composed of buffer system like phosphate and acetate and other with different pH ranged from 2 to 7 and organic modifier like methanol and acetonitril were tested to provide sufficient selectivity and sensitivity in short separation time. The best chromatographic separation occurred on cyanopropyl column with a mobile phase consisting of 10 mM KH_2PO_4 (pH 6.0) – methanol (70:30, v/v) at a flow rate of 1 mL min^{-1} and UV detection at 225 nm (Figs. 1 and 2).

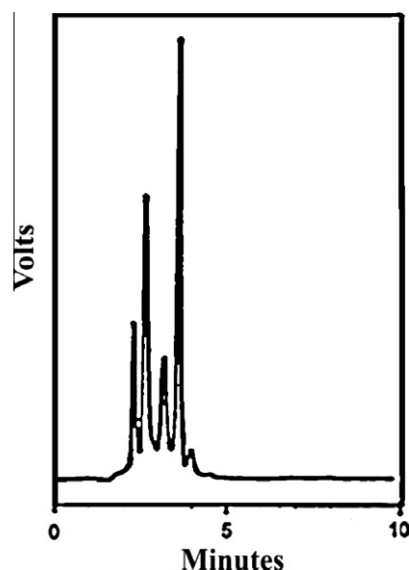


Figure 1 HPLC chromatogram of 20 µL injection of human blank plasma samples.

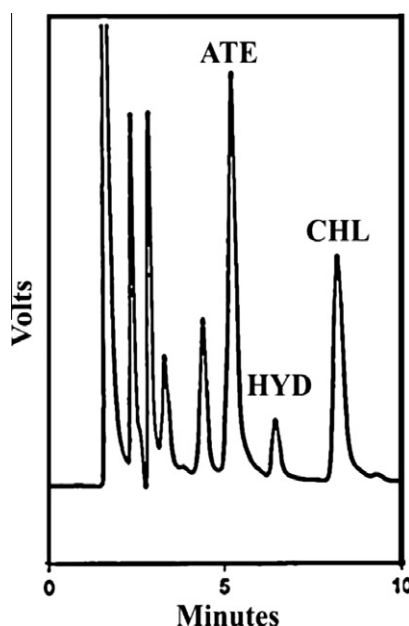


Figure 2 HPLC chromatogram of 20 µL injection of atenolol (ATE), chlorthalidone (CHL), and hydrochlorothiazide (HYD) as IS spiked in human plasma.

The influence of both organic modifier (methanol) concentration and pH was carefully studied. Increasing organic modifier concentration not only improves peak shape and decreasing the run time but also decreasing method specificity due to the interference of the atenolol peak with endogenous biological substance. Decreasing of organic modifier concentration below 20% resulted in high specificity with regard to the separation of the studied drugs from endogenous biological substances, and more retained of the drug on the column that led to excessive tailing of eluting peaks and long run time.

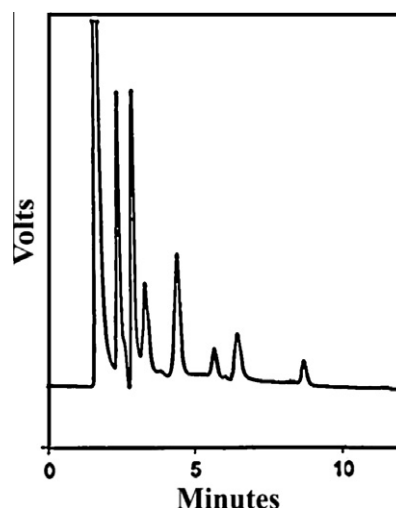


Figure 3 HPLC chromatogram of 20 µL injection of patient plasma sample, 4 h following oral administration of Bolkium Diu® tablet (100 mg atenolol + 25 mg chlorthalidone).

Variation of pH played an important role in the separation process. At pH (<3.5), atenolol became more retained on the column and eluted with some degree of overlapping with hydrochlorothiazide (IS). At pH (3.5–5.0) good resolution was obtained with better peak shape for the three drugs but atenolol was eluted interfering with endogenous biological substances. pH (6.0) was chosen as the optimum value both for the resolution of the drugs from endogenous biological substances and better peak shape and reasonable run time (< 10 min).

Hydrochlorothiazide was considered as best (IS) applied for determination of atenolol and chlorthalidone when compared to other substances such as paracetamol, caffeine, theophylline and guaifensine owing to retention characters that are not affected seriously by change of pH, therefore, it was eluted with reasonable resolution from atenolol and chlorthalidone, and absorbance characters that show high absorbance at the chosen wavelength (225 nm) that increase the sensitivity of the method.

The proposed method succeeds in the determination of the studied mixture in spiked plasma sample and also in real patient plasma sample, 4 h following oral administration of Bolkium Diu® tablet (100 mg atenolol and 25 mg chlorthalidone) (Figs. 2 and 3).

4. Validation of the proposed method

4.1. Specificity and selectivity

Before the preparation of the pooled calibration standards and QC samples, six lots of blank plasma were screened for matrix effects or interferences. The interference from individual blank plasma in the LC-UV chromatograms at the retention times of the studied drug and IS with or without both drugs and IS was investigated to ensure the specificity of the method. Each sample of six blank plasmas was eluted three times individually and they found to be free of co-eluting peaks at the retention time range of drugs and IS (Fig. 1).

Table 1 Assay parameters and regression characteristic atenolol and chlorthalidone determined by the proposed HPLC method.

Parameters	Atenolol	Chlorthalidone
Linearity range ($\mu\text{g mL}^{-1}$)	0.1–10	0.1–10
Detection limit ($\mu\text{g mL}^{-1}$)	0.06	0.038
Quantitation limit ($\mu\text{g mL}^{-1}$)	0.2	0.129
Regression equation (y^*)		
Number of experiments (n)	8	8
Slope (b)	0.189	0.529
Standard deviation of slope	0.005	0.0091
Relative standard deviation of slope	2.64	1.7
Intercept (a)	0.0524	0.022
Standard deviation of intercept	0.026	0.0365
Correlation coefficient (r)	0.998	0.9991
Standard error of regression	0.048	0.084

* $Y = a + bC$, where C is the concentration ($\mu\text{g/mL}$) and Y is the peak area.

Table 2 Recovery of atenolol and chlorthalidone from plasma determined by the proposed HPLC method.

Concentration ($\mu\text{g mL}^{-1}$)	Recovery % (mean \pm RSD)	
	Atenolol	Chlorthalidone
0.1	94.54 \pm 7.21	93.12 \pm 6.4
1	95.3 \pm 4.1	98.47 \pm 5.7
10	98.24 \pm 6.8	94.01 \pm 3.5

4.2. Linearity of calibration curves and lower limit of quantitation

During prestudy validation, the calibration curves were defined in three runs based on triplicate assays of the spiked blank plasma samples. The regression parameters of slope, intercept and correlation coefficient were calculated by a least-squares linear-regression analysis (Table 1). Good linearity was obtained over the concentration range of 0.1–10 $\mu\text{g mL}^{-1}$. A typical linear regression equation, the lower limit of quantitation (LLOQ) and limit of detection (LD) and other parameters for the determination of atenolol and chlorthalidone in human plasma were listed in Table 1. The linearity of calibration curves was validated by the high value

of correlation coefficient and the intercept value, which was not statistically ($P = 0.05$) different from zero (Table 1).

4.3. Recovery

The absolute recoveries of the studied drugs were determined by comparing the peak area ratio of the QC sample spiked in human plasma and defined in three runs with those of post extracted plasma blanks fortified with the known amount of analytes. The results in Table 2 indicate high ability of the proposed method to recover the studied drugs from human plasma.

4.4. Precision and accuracy

The precision and accuracy of the method were assessed by analyzing six replicate QC samples at the low, medium and high concentration levels. The accuracy of the method was determined by calculating relative error (RE) and the precision by calculating RSD. Table 3 summarizes the precision and accuracy on each of three assays for two drugs in human plasma with accuracy ranging from -8% to 6.2% (RE) of nominated values and the precision ranging from 0.236% to 6.5% (RSD) over the three concentration levels evaluated.

4.5. Stability

The stability of the studied drugs in human plasma was assessed by analyzing six replicate QC samples at the low, medium and high concentration levels at ambient temperature over 24 h (bench top stability). The measured concentrations of the drugs in these QC samples sitting at room temperature for 24 h were compared with that obtained with the corresponding QC sample freshly prepared and proceed immediately. The results in table indicate that the studied drugs were stable for at least 24 h in human plasma when stored at ambient temperature. On the other hand, QC samples experiencing three freeze–thaw cycles (freeze–thaw stability) were analyzed together. The results indicate the stability of the studied drug in human plasma over three freeze–thaw cycles (Table 4). Also the studied drug showed the stability in human plasma when stored at -20°C for one month (where the whole samples were frozen, thawed and completely analyzed as mentioned) as long term stability when compared with the freshly prepared sample.

Table 3 Intra- and inter-day precision and accuracy for the determination of atenolol and chlorthalidone in human plasma.

Add concentration ($\mu\text{g mL}^{-1}$)	Intra-day			Inter-day								
	0.1	1	10	1st day			2nd day			3rd day		
				0.1	1	10	0.1	1	10	0.1	1	10
<i>Atenolol</i>												
Mean	0.098	1.06	9.89	0.092	0.983	9.75	0.102	0.968	10.14	0.107	0.983	10.24
SD	0.006	0.058	0.321	0.003	0.062	0.45	0.006	0.04	0.371	0.007	0.052	0.54
RSD %	6.122	5.472	3.246	3.261	6.307	4.615	5.882	4.132	3.659	6.542	5.290	5.273
RE %	−2	6	−1.1	−8	−1.7	−2.5	2	−3.2	1.4	7	−1.7	2.4
<i>Chlorthalidone</i>												
Mean	0.0924	0.958	10.21	0.102	1.043	10.14	0.1062	0.984	9.75	0.0985	1.04	9.56
SD	0.005	0.018	0.27	0.004	1.04	0.54	0.007	0.035	0.023	0.002	0.028	0.121
RSD %	5.411	1.879	2.644	3.922	1.04	5.325	6.591	3.557	0.236	2.030	2.692	1.266
RE %	−7.6	−4.2	2.1	2	1.04	1.4	6.2	−1.6	−2.5	−1.5	4	−4.4

Table 4 Summary of stability of atenolol and chlorthalidone in human plasma at varying condition.

Add concentration ($\mu\text{g mL}^{-1}$)	Theoretical concentration					
	Atenolol			Chlorthalidone		
	0.1	1	10	0.1	1	10
<i>(a) Three freeze-thaw cycles</i>						
Mean	0.0924	1.07	10.17	0.0991	0.955	10.32
SD	0.003	0.104	0.26	0.01	0.081	0.331
RSD %	3.2468	9.7196	2.5565	10.091	8.4817	3.2074
RE %	-7.6	7	1.7	-0.9	-4.5	3.2
<i>(b) Room temperature for 24 h</i>						
Mean	0.0891	1.04	10.04	0.0954	1.075	9.56
SD	0.004	0.12	0.171	0.005	0.054	0.231
RSD %	4.4893	11.538	1.7032	5.2411	5.0233	2.4163
RE %	-10.9	4	0.4	-4.6	7.5	-4.4
<i>(c) Re-injection after 30 day at -20°C</i>						
Mean	0.091	1.113	10.22	0.0964	0.987	10.05
SD	0.008	0.176	0.251	0.008	0.107	0.075
RSD %	8.7912	15.813	2.456	8.2988	10.841	0.7463
RE %	-9	11.3	2.2	-3.6	-1.3	0.5

4.6. Comparison of the proposed method with the reported methods

When comparing between the proposed method and the published methods for simultaneous determination of the binary mixture (Elshanawane et al., 2009; El-Gindy et al., 2008; Giachetti et al., 1997) found that:

- The suggested method is highly selective when compared to our previous method that failed to separate atenolol from plasma matrix (Elshanawane et al., 2009).
- The proposed method utilizes proteins precipitation with acetonitril as the only sample preparation involved prior to reverse phase-HPLC rather than the other method that requiring many steps before injection (El-Gindy et al., 2008) or depending on liquid-liquid extraction that need long time and effort for sample preparation before injection, and also the possibility of drug lost during preparation is high (Giachetti et al., 1997).
- The run time of the proposed method is short when compared to the published method (Giachetti et al., 1997) that needs 20 min to elute the two drugs.

5. Conclusion

The method developed is a simple, rapid, accurate, and reliable procedure for the analysis of atenolol and chlorthalidone in human plasma, meeting all requirements for the validation of an analytical methodology. It is adequate to monitor patients receiving therapeutic dosage of the drugs.

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